# *In Vitro* Inhibition of the Diphenolase Activity of Tyrosinase by Insecticides and Allelochemicals in *Micromelalopha troglodyta* (Lepidoptera: Notodontidae)[1]

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**Abstract** Tyrosinase is a copper enzyme and plays a key role in normal insect development. We studied the *in vitro* inhibitory effects of selected insecticides and allelochemicals on the diphenolase activity of tyrosinase in *Micromelalopha troglodyta* (Graeser) (Lepidoptera: Notodontidae). Two pyrethriods (cyfluthrin and deltamethrin) and 3 other insecticides (hexaflumuron, abamectin and imidacloprid) were the least inhibitory, whereas 5 organophosphates (triazophos, malathion, chlorpyrifos, omethoate and profenofos), 1 carbamate (methomyl), 4 pyrethriods (fenpropathrin, beta-cypermethrin, bifenthrin and lambda-cyhalothrin), 1 organochlorine (endosulfan), 2 allelochemicals (tannic acid and 2-tridecanone) and 4 other insecticides (emamectin benzoate, fipronil, acetamiprid and pyridaben) were moderately inhibitory. Three chemicals (quercetin, phenyl thiourea and phoxim) were the most potent inhibitors of the enzymes among all compounds tested and inhibited the diphenolase activity of tyrosinase *in vitro* in a dose-dependent manner. Furthermore, phenyl thiourea, phoxim and quercetin showed neither typical competitive nor noncompetitive binding to the substrate, with *Ki* of 0.13 μM, 49.30 μM and 37.71 μM, respectively.

**Key Words** diphenolase activity, tyrosinase, *Micromelalopha troglodyta*, allelochemicals, insecticides, inhibition

It is well known that tyrosinase (EC 1.14.18.1), also known as phenoloxidase, is a copper enzyme that is widely distributed among microorganisms, plants, insects and animals (Yang et al. 2005, Liu et al. 2006). It is critical in biological processes such as vertebrate pigmentation and the browning of fruits and vegetables. It is the key enzyme involved in melanin formation in melanocytes. Tyrosinase catalyzes both the hydroxylation of monophenols and the oxidation of *o*-diphenols into *o*-quinones. Further, it is involved in the formation of pigments such as melanin (Hopkins and Kramer 1992, Xiao et al. 2008). In insects, tyrosinase is a widely distributed enzyme that plays important roles in normal developmental processes, such as cuticular tanning, sclerization, wound healing, production of opsonins, and encapsulation and nodule formation for defense against foreign pathogens. Diphenolase is the pivotal insect enzyme in the oxidation of catecholamine to their respective quinines, which are then further metabolized to either melanin or crosslink proteins in sclerotin (Iwama and Ashida

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1986, Hopkins and Kramer 1992, Chase et al. 2000, Ma and Kanost 2000, Wang et al. 2005).

The small prominent, *Micromelalopha troglodyta* (Graeser) (Lepidoptera: Notodontidae), is an important pest of poplar in China. Insecticides are widely used for its control. This pest has developed resistance to almost all classes of insecticides, such as pyrethroids, organophosphates and carbamates. There are many reports on insect resistance due to tryosinase abundance (Gao et al. 1997). It is possible that inhibition of tyrosinase could lead to abrogation of insect defense mechanisms or abnormal body softening, both of which could enhance control. Thus, tyrosinase may be regarded as a potential candidate for the novel insecticide development. But, little is known about the biochemical properties of tryosinase in *M. troglodyta*. To better understand the interaction of tryosinase with insecticides and allelochemicals, we studied their *in vitro* inhibitory effects on tryosinase activity in *M. troglodyta*. We also attempted to identify some potential tryosinase inhibitors which might be used as synergists to enhance the toxicity of insecticides.

## Materials and Methods

**Chemicals.** Bovine serum albumin (BSA), 2-tridecanone and tannic acid (>99%) were purchased from Sigma Chemical Co. (St. Louis, MO), quercetin (99%) from Shanghai chemical stock (Shanghai, China). Content and manufacturers of insecticides are shown in Table 1. Allelochemicals and insecticides were dissolved in absolute acetone. All other chemicals were of analytical grade and purchased from commercial sources.

**Insects.** Small prominents were collected from Mingguang of Anhui Province, China, in 2007. They were reared in the conditioned room maintained at 26 ± 1°C, 75% RH and a 12:12 L:D photoperiod. Fifth-instar newly-molted larvae were used in all studies.

**Preparation of cytosolic fractions.** Ten fifth-instar larvae were homogenized in 5 mL of ice-cold phosphate buffer (pH 7.5, 0.1M) after peritrophic membranes and associated midgut contents were removed. The homogenate was centrifuged at 27,500 g for 20 min at 4°C. The supernatants were used to determine the enzyme activity after being filtered through 3 layers of cellulose filter paper (grade 3, Whatman, Middlesex, UK).

**Diphenolase activity of tyrosinase assay.** The assay method was that described by Liang et al. (2003) with the following modifications. The assay mixture contained 5 mM catethol. The assay was initiated by the addition of 70 μL enzyme; the absorbance at 420 nm was monitored for 2 min. Controls without enzyme always accompanied each assay. Enzyme activity was expressed as OD/min at 25°C, and the specific activity as OD/min/mg protein. The method of Bradford (1976), with BSA as a standard, was used for protein quantification.

**Inhibition of insecticides and allelochemicals.** Inhibition of the diphenolase of tyrosinase was determined in assays containing 5 mM catethol and various insecticides (0.1 mM) or allelochemicals (0.1mM) as inhibitors. All assays, including controls, contained 1% acetone. All assays were run in triplicate.

Dose-dependent inhibition of the diphenolase activity of tyrosinase was studied with fixed concentrations of 5 mM catethol by adding 30 μL of insecticides or allelochemicals dissolved in acetone at various concentrations to the incubating reaction mixtures. The $I_{50}$ values, concentrations of inhibitors required to reduce the reaction rate by 50%,

**Table 1. Content and manufacturers of insecticides**

| Insecticides | Content (%) | Manufacturer | Location |
|---|---|---|---|
| Organophosphate | | | |
| Triazophos | 92.0 | Jiangxi Kaifeng Chemical Co., Ltd | Jiangxi, China |
| Malathion | 95.0 | Hebei Shiji Pesticide Co., Ltd | Hebei, China |
| Chlorpyrifos | 97.0 | Dow AgroSciences LLC | Indiana, USA |
| Phoxim | 99.0 | Tianjin Pesticide Co., Ltd | Tianjin, China |
| Omethoate | 92.0 | Hangzhou Qingfeng Agrochemicals Co., Ltd | Zhejiang, China |
| Profenofos | 90.0 | Tianjin Pesticide Co., Ltd | Tianjin, China |
| Carbamate | | | |
| Methomyl | 98.0 | Hubei Sanongda Co., Ltd | Hubei, China |
| Pyrethriod | | | |
| Fenpropathrin | 92.0 | Shandong Dacheng Pesticide Co., Ltd | Shandong, China |
| Beta-cypermethrin | 97.0 | Shandong Dacheng Pesticide Co., Ltd | Shandong, China |
| Bifenthrin | 97.0 | Jiangsu Yangnong Chemical Group Co., Ltd | Jiangsu, China |
| Lambda-cyhalothrin | 95.0 | Jiangsu Huangma Pesticide & Chemical Co., Ltd | Jiangsu, China |
| Deltamethrin | 99.0 | Jiangsu Yangnong Chemical Group Corp., Ltd | Jiangsu, China |
| Cyfluthrin | 92.0 | Jiangsu Yangnong Chemical Group Corp., Ltd | Jiangsu, China |
| Organochlorine | | | |
| Endosulfan | 90.0 | Jiangsu Rudong Pesticide Co., Ltd | Jiangsu, China |
| Other insecticides | | | |
| Phenyl thiourea | 99.0 | Shanghai No.1 chemical factory | Shanghai, China |
| Hexaflumuron | 95.0 | East Romble Agrochem (Shan Dong) Co., Ltd | Shandong, China |
| Emamectin benzoate | 90.0 | Zhejiang Qianjiang Biochemical Co., Ltd | Zhejiang, China |

**Table 1. Continued**

| Insecticides | Content (%) | Manufacturer | Location |
|---|---|---|---|
| Abamectin | 95.3 | Shandong Jingbo Agrochemicals Co., Ltd | Shandong, China |
| Fipronil | 90.0 | Anhui Huaxing Chemical Industry Co., Ltd | Anhui, China |
| Imidacloprid | 95.0 | Hubei Sanongda Co., Ltd | Hubei, China |
| Acetamiprid | 96.0 | Qingdao Haili'er Medicine Co., Ltd | Shandong, China |
| Pyridaben | 95.0 | Shandong Sino-Agri United Biotechnology Co., Ltd | Shandong, China |

were determined by linear regression of the inhibition percent on the log of the inhibitor concentration. The experiment was performed in triplicate for each inhibitor.

**Kinetics of tyrosinase inhibition.** Kinetics of tyrosinase inhibition by phenyl thiourea, phoxim and quercetin were determined in assays containing various concentrations of catethol and a fixed concentration of each inhibitor. The results were presented on double-reciprocal Lineweaver-Burk plots. The inhibitory constant, *K*i, the equilibrium constant for dissociation of the enzyme-inhibitor complex, was determined from plots of reciprocal velocity versus reciprocal concentration (Lineweaver-Burk plots) (Dixon and Webb 1979).

## Results

The inhibition of the diphenolase activity of tyrosinase by insecticides and allelochemicals is shown in Table 2. Three inhibitors (quercetin, phenyl thiourea and phoxim) were the most potent inhibitors tested, inhibiting more than 65% of tyrosinase activity at a final concentration of 0.1 mM. Among the inhibitors tested, 5 organophosphates (triazophos, malathion, chlorpyrifos, omethoate and profenofos), 1 carbamate (methomyl), 4 pyrethriods (fenpropathrin, beta-cypermethrin, bifenthrin and lambda-cyhalothrin), 1 organochlorine (endosulfan), 2 allelochemicals (tannic acid and 2-tridecanone) and 4 other insecticides (emamectin benzoate, fipronil, acetamiprid and pyridaben) were moderately inhibitory, whereas 2 pyrethriods (cyfluthrin and deltamethrin) and 3 other insecticides (hexaflumuron, abamectin and imidacloprid) were the least inhibitory.

The sensitivity of tyrosinase activity to 3 inhibitors (phenyl thiourea, phoxim and quercetin) was also assessed. Phenyl thiourea, phoxim and quercetin inhibited the diphenolase activity of tyrosinase *in vitro* in a dose-dependent manner (Fig. 1). The $I_{50}$ values of these 3 inhibitors for tyrosinase activity in *M. troglodyta* larvae are summarized in Table 3, with $I_{50}$ values ranging from $3.34 \times 10^{-8}$ M to $7.25 \times 10^{5}$ M, phenyl thiourea being the most potent inhibitor.

The results of kinetic analyses of tyrosinase inhibition by phenyl thiourea, phoxim and quercetin using Lineweaver-Burk plots are summarized in Fig. 2. Phenyl thiourea, phoxim and quercetin showed neither typical competitive nor noncompetitive binding to the substrate, with *K*i of 0.13 μM, 49.30 μM and 37.71 μM, respectively.

**Table 2. Inhibition of the diphenolase activity of tyrosinase by insecticides and allelochemicals in *M. troglodyta* larvae**

| Inhibitor (0.1 mM) | % Inhibition (means ± SD) |
|---|---|
| Organophosphate | |
| Triazophos | 24.62 ± 2.18 fghi |
| Malathion | 10.00 ± 1.63 jk |
| Chlorpyrifos | 26.28 ± 3.89 fgh |
| Phoxim | 68.46 ± 4.16 c |
| Omethoate | 19.33 ± 1.20 ghij |
| Profenofos | 25.79 ± 5.78 fghi |
| Carbamate | |
| Methomyl | 12.20 ± 1.35 j |
| Pyrethriod | |
| Fenpropathrin | 10.00 ± 2.18 jk |
| Beta-cypermethrin | 14.34 ± 1.33 j |
| Bifenthrin | 16.05 ± 0.79 ij |
| Lambda-cyhalothrin | 34.07 ± 1.18 def |
| Cyfluthrin | −6.40 ± 1.46 l |
| Deltamethrin | −3.93 ± 0.88 l |
| Organochlorine | |
| Endosulfan | 12.67 ± 2.35 j |
| Other insecticides | |
| Phenyl thiourea | 96.20 ± 2.09 b |
| Hexaflumuron | 1.10 ± 0.52 kl |
| Emamectin benzoate | 16.36 ± 2.86 hij |
| Abamectin | −7.12 ± 1.36 l |
| Fipronil | 10.77 ± 0.54 jk |
| Imidacloprid | −2.88 ± 1.36 l |
| Acetamiprid | 33.85 ± 2.15 def |
| Pyridaben | 40.80 ± 1.71 d |
| Allelochemical | |
| Quercetin | 138.33 ± 11.44 a |
| Tannic acid | 28.27 ± 1.90 efg |
| 2-tridecanone | 36.71 ± 2.26 de |

The data were analyzed using analysis of variance (ANOVA). The difference is significant if $P < 0.05$. Means within a column followed by the same small letter are not significantly different.

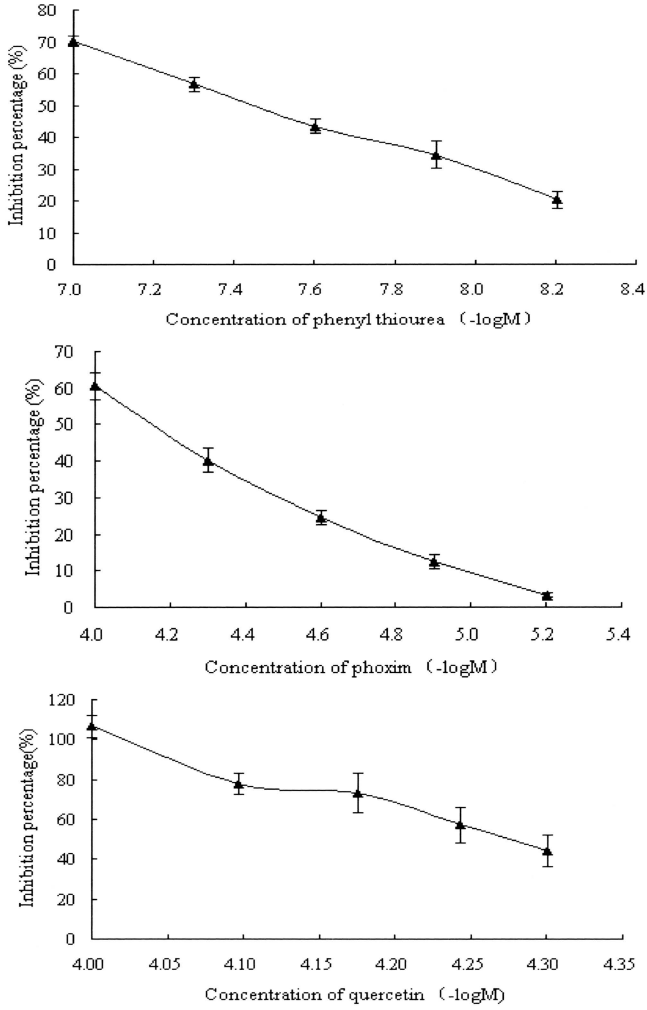


**Fig. 1. *In vitro* inhibition of the diphenolase activity of tyrosinase by three inhibitors in *M. troglodyta* larvae.**

## Discussion

Tyrosinase plays a key role in normal insect development, so it may be possible to control pests by inhibiting or disturbing the enzyme. This maybe a useful basis for the development of novel insecticides (Wang et al. 2005). *In vitro* inhibition studies are useful for understanding the potential as insecticides. In the present study, we found that most of inhibitors tested inhibited the *in vitro* diphenolase activity of tyrosinase in *M. troglodyta* at the concentration of 0.1 mM. Our results differ from previous studies. For example, organophosphates (malathion and chlorpyrifos) and pyrethroids

**Table 3. $I_{50}$ values of inhibitors for the diphenolase activity of tyrosinase in *M. troglodyta* larvae**

| Inhibitor | $I_{50}$ ($1 \times 10^{-5}$ M) (means ± SD) |
|---|---|
| Phenyl thiourea | $(3.34 \pm 0.35) \times 10^{-3}$ c |
| Phoxim | 7.25 ± 0.46 a |
| Quercetin | 5.24 ± 0.57 b |

The data were analyzed using analysis of variance (ANOVA). The difference is significant if $P < 0.05$. Means within a column followed by the same small letter are not significantly different.

(beta-cypermethrin) were moderately inhibitory to the diphenolase activity of tyrosinase in *M. troglodyta*; whereas these 3 insecticides were noninhibitory to the diphenolase activity of tyrosinase in *Musca domestica* (L.) (Liu et al. 2004). Abamectin and imidacloprid were noninhibitory to the diphenolase activity of tyrosinase in *M. troglodyta*; whereas these 2 insecticides demonstrated low inhibition to the diphenolase activity of tyrosinase in *M. domestica* (Liu et al. 2004). Further, our results showed phoxim produced >68% inhibition of the diphenolase activity of tyrosinase in *M. troglodyta* at the concentration of 0.1 mM. Compared with previous reports, the inhibitory effect of phoxim was very high. For example, Liu et al. (2004) showed that phoxim showed 5.3% inhibition of the diphenolase activity of tyrosinase in *M. domestica* at the concentration of 15.6 mM. These results suggest that isoenzymes of tyrosinase may affect susceptibility to insecticides.

Our results show that the $I_{50}$ value of phenyl thiourea for the diphenolase activity of tyrosinase in *M. troglodyta* larvae is $3.34 \times 10^{-8}$ M, being the most potent inhibitor. Liang et al. (2003) reported the $I_{50}$ value of phenyl thiourea for the diphenolase activity of tyrosinase from both the resistant and susceptible strains of *Plutella xylostella* (L.) showed no obvious differences ($1.18 \times 10^{-6}$ M to $1.28 \times 10^{-6}$ M, respectively). Liu et al. (2004) reported the $I_{50}$ value of phenyl thiourea for the diphenolase activity of tyrosinase in *M. domestica* was $1.50 \times 10^{-7}$ M. These results showed that phenyl thiourea is an effective tyrosinase inhibitor. Hopefully, this work will provide the basis for the design of effective, selective tyrosinase inhibitors and the development of novel candidate insecticides.

Our kinetic analysis indicated that quercetin showed neither typical competitive nor noncompetitive interaction with the substrate in *M. troglodyta*. However, the inhibition of *Spodoptera exigua* (Hübner) tyrosinase by quercetin was competitive (Luo et al. 2005). These results also indicate that inhibitors have different reaction modes to different isoenzymes. Inhibition mechanisms may differ significantly even for isoenzymes very similar in structure.

Some allelochemicals might be potent inhibitors of tyrosinase activity in insects. Quercetins are plant phenols; potent inhibition was observed in our study. The $I_{50}$ value of quercetin for the diphenolase activity of tyrosinase in *M. troglodyta* larvae is $5.24 \times 10^{-5}$ M, being more potent inhibitor of tyrosinase activity than phoxim. This result was consistent with other observations. For example, Luo et al. (2005) reported that the $I_{50}$ value of quercetin for the diphenolase activity of tyrosinase in *S. exigua* is $8.70 \times 10^{-5}$ M. Our results showed that quercetin had the potential of serving as a synergist for enhancing the toxicity of insecticides in *M. troglodyta*. However, its application as a synergist in insect control requires further investigation.

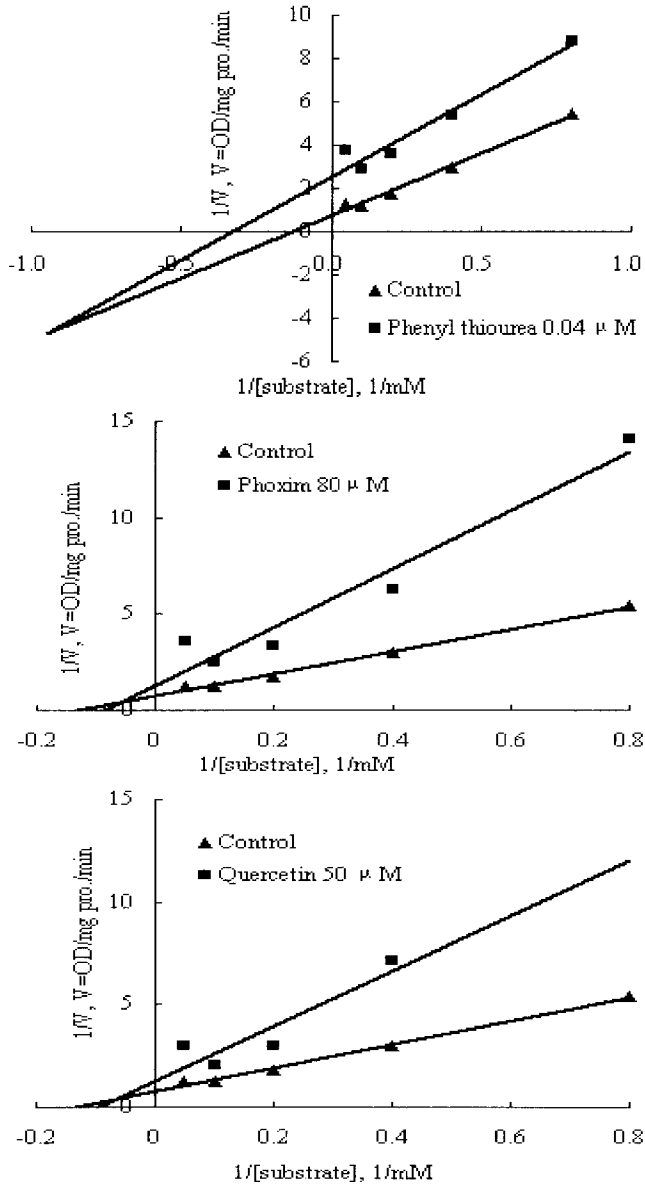


**Fig. 2. Kinetics of tyrosinase inhibition by three inhibitors in *M. troglodyta* larvae.**

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